Protective potential of an attenuated *Pasteurella multocida*, which expresses only the N-terminal truncated fragment of *P. multocida* toxin

Jayoung Seo, Semi Lee, Hyoju Pyo, Jaeil Lee, Taejung Kim

Abstract

Pasteurella multocida serogroup D causes progressive atrophic rhinitis in pigs and produces a potent, intracellular, mitogenic toxin known as *P. multocida* toxin (PMT), which is encoded by the *toxA* gene. Highly toxic to cells, PMT is a poor antigen and becomes more immunogenic after its native structure has been destroyed. Previously, we found that the N-terminal fragment of PMT (N-PMT) can induce a strong immune response that is protective against wild-type challenge. Here, an attenuated *P. multocida* mutant expressing only N-PMT was developed and its protective effect was evaluated. The mutant provides protective immune responses against bacterial and toxin challenges, and so is a good live vaccine candidate.

Résumé

Pasteurella multocida *sérogroupe D est responsable de la rhinite atrophiante progressive du porc et produit une toxine intracellulaire puissante et mitogène dénommée toxine de* P. multocida *(PMT) qui est codée par le gène* toxA. *PMT est fortement toxique pour les cellules mais est un pauvre antigène et devient immunogène après que sa structure primaire ait été détruite. Précédemment, nous avons trouvé que le fragment N-terminal de PMT (N-PMT) peut induire une forte réponse immune qui est protectrice contre une infection défi avec une souche sauvage. Ici, un mutant atténué de* P. multocida *exprimant uniquement N-PMT a été développé et son effet protecteur évalué. Le mutant induit des réponses immunes contre une infection défi avec la bactérie et l'administration de toxine, et serait ainsi un bon candidat comme vaccin vivant.*

(Traduit par Docteur Serge Messier)

Introduction

Pasteurella multocida-induced pneumonia and progressive atrophic rhinitis (PAR) are widespread diseases that cause growth retardation and a reduction in the efficiency of feed utilization among grower-finisher pigs (1–3). *Pasteurella multocida* toxin (PMT), a monomeric 146 kDa protein encoded by the *toxA* gene, is produced by some *P. multocida* serotype A and D strains (4). A poor antigen, PMT becomes more immunogenic after its native structure has been destroyed (5). Partially truncated proteins have been predicted to be good antigens (6). In a previous study, vaccination with a mixture of 3 recombinant fragments of PMT with/without inclusion of intact *P. multocida* resulted in high levels of neutralizing antibody (Ab) and protection against PMT challenge (6).

Attenuation of *P. multocida* can be achieved by the abrogation of the appropriate metabolic gene. In a previous study, an *aroA* mutant of *P. multocida* successfully protected calves against challenge with the pathogenic wild type (7). However, PMT was not involved since the toxin is expressed only in serotypes A and D in pigs (4). Previously, we demonstrated that none of the mice vaccinated with a *toxA* knock-out mutant that does not produce PMT were capable of surviving challenge with the wild type (8) indicating that mouse Abs against outer structural and/or inner cytosolic proteins of *P. multocida* are not protective. So, it is clear that the targeting of the protection against *P. multocida* serotypes A and/or D should be focused on PMT.

We have previously shown that the N-terminal fragment of PMT (N-PMT, amino acids 1-390) is the most immunogenic portion of the protein, and that N-PMT is partially protective for mice against wild type challenge (9). To clarify whether N-PMT expressed in vivo can induce protective immunity against bacterial and toxin challenge, a *P. multocida* mutant capable of expressing only N-PMT instead of the intact toxin was developed and its protective effect was evaluated.

Materials and methods

Escherichia coli and plasmids

Escherichia coli JM109 (Invitrogen, Carlsbad, California, USA) was used to propagate the plasmid construct. The pGEM®-T easy vector (Promega, Madison, Wisconsin, USA) was used for cloning procedures. *Escherichia coli* manipulations were performed according to the manufacturer's instructions. Standard DNA and protein manipulations were carried out as previously described (10,11). Red

Biotherapy Human Resources Center, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea.

Address all correspondence to Dr. Taejung Kim; telephone: 82-62-530-2858; fax: 82-62-530-2857; e-mail: tjkim@chonnam.ac.kr

Dr. Kim's current address is 300 Yongbong-dong, Buk-gu, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea.

Received July 13, 2008. Accepted December 10, 2008.

Table I. Nucleotide sequences of the primers used in this study (GenBank reference numbers: AF240778 for *toxA* gene in *P. multocida* and AY048744 for *kanR* gene in pKD13)

Primers		Nucleotide sequence (5′– 3′)
P1	*toxA*-N-F	GCGC CTCGAG* ATGAAAACAAAACATTTTTT
P2	*toxA*-N-R	GCGC AGATCT* GAGTAATGAAGAGCATAGT
P3	*toxA*-C-F	GCGC GGTACC* ATTGACTTTTTCCTAAATAA
P4	*toxA*-C-R	AATT GGATCC* TTATAGTGCTCTTGTTAAGC
P5	kan^R-F	GGCC AGATCT* ATGATTGAACAAGATGGAT
P6	kan^R-R	AATT GGTACC* TCAGAAGAACTCGTCAAGA

*Underlined: enzyme sites.

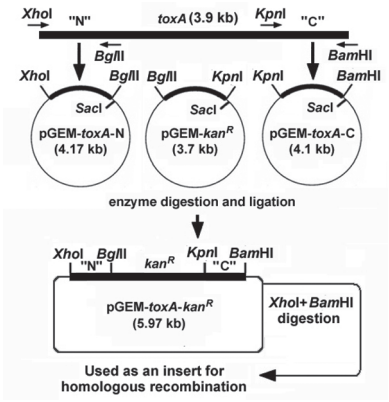

Figure 1. Diagram of cloning strategy. After cloning, an insert was prepared by digesting pGEM-*toxA*-kan^R with *Xho*I and *Bam*HI restriction enzymes.

helper plasmid pKD46 (12), which expresses λ Red recombinase, was used to allow the homologous recombination of linear DNA in strain JM109. pKD13 (12) was used as a template for the generation of kanamycin resistance gene (kan^R). pKD46 is a temperature sensitive replicon, thus allowing its easy elimination at 43°C.

Cloning of *toxA* and kan^R fragments

Pasteurella multocida type D was originally obtained from the National Veterinary Research & Quarantine Service, Korea. The N-terminal (amino acids 1–390) and C-terminal (amino acids 921–1285) regions of *toxA* were amplified using *P. multocida* genomic DNA as a template. For the selection of knock-out colonies, kan^R was used for transformant selection, which was amplified by polymerase chain reaction (PCR) using pKD13 as a template. Six PCR primers (P1–P6) were designed using the Gene Runner software program (Hastings Software, Hastings, New York, USA) from the nucleotide sequences in the GenBank database (Table I). The amplified DNA products were electrophoresed on a 1.2% (w/v) agarose gel, purified using a PCR purification kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions, and cloned into a pGEM®-T easy vector (Qiagen) to generate pGEM-*toxA*-N, pGEM-*toxA*-C, and pGEM-kan^R (Figure 1). The construct was transformed into chemically competent *E. coli* JM109. The transformants were selected and the mini-scale isolation of the plasmid DNA was used to prepare the recombinant plasmid for sequencing on the plasmid DNA QIAprepSpin Mini Kit (Qiagen). *Eco*RI restriction analysis and DNA sequencing confirmed the presence and orientation of each gene or gene segment. DNA sequencing reactions were performed using an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, California, USA).

Cloning strategy

Two constructs (pGEM-*toxA*-N and pGEM-kan^R) were digested with *Bgl*II and *Sac*I, and the kan^R gene was inserted into the *Bgl*II+*Sac*I site of pGEM-*toxA*-N, generating pGEM-*toxA*-N-kan^R. pGEM-t*oxA*-C was digested with *Kpn*I and *Sac*I and inserted into *Kpn*I+*Sac*I site of pGEM-*toxA*-N-kan^R, generating pGEM-*toxA*-kan^R. A linear 2.97 kb DNA fragment (*toxA*-N-kan^R-*toxA*-C) produced by digestion with *Xho*I and *Bam*HI was used as an insert (100 ng/μL) for homologous recombination. The cloning strategy is depicted in Figure 1.

Gene disruption by homologous recombination

A 5-mL volume of a fresh overnight *P. multocida* culture was inoculated into 500 mL Brain-Heart Infusion (BHI). Cells were grown to an optical density at 600 nm (OD_{600}) of approximately 0.5, chilled on ice for 20 min, and centrifuged at 4000 × *g* for 15 min at 4°C. The supernatant was removed and the pellet was concentrated 100-fold and washed 3 times with ice-cold 10% glycerol. The final preparation represented the competent cells.

For the induction of λ Red recombinase, competent cells were transformed with pKD46 using a Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, California, USA) according to the manufacturer's instructions. Transformants carrying pKD46 were grown in 5 mL SOB containing 10 mM each of ampicillin and L-arabinose (final) at 30°C to an OD_{600} of approximately 0.5, and then made electro-competent by concentrating 100-fold and washing 3 times with ice-cold 10% glycerol. Electro-competent cells (40 μL) were transformed with 1 μL (100 ng) of insert DNA (*toxA*-N-kan^R-*toxA*-C) by electroporation according to the manufacturer's instructions. Knock-out mutants were selected on LB plates containing 50 μg/mL kanamycin.

PCR verification

Three PCR procedures were used to show that mutants had the correct genomic structure. Genomic DNA from a mutant colony was prepared (50 ng/μL) and used as a template. The expected sizes and targets of the 3 PCR approaches are represented in Figure 2.

Table II. Experimental design for toxigenicity test and protection study. Mice in groups 1 and 2 were vaccinated with live PMT mutant *P. multocida* bacterial culture, while controls (groups 3 and 4) with PBS. For challenge study, group 2 and 4 were challenged with W/T *P. multocida*, while group 1 and 3 with W/T bacterial lysate

Group	Route	Dosage per mouse	Trial number	Challenge with
1	Intraperitoneal	PMT mutant (1×10^7 cells)	12	Bacterial lysate 500 μL
2	Intraperitoneal	PMT mutant (1×10^7 cells)	12	Bacterial culture (1×10^7 cells)
3	Intraperitoneal	PBS 100 μL	12	Bacterial lysate 500 μL
4	Intraperitoneal	PBS 100 μL	12	Bacterial culture (1×10^7 cells)

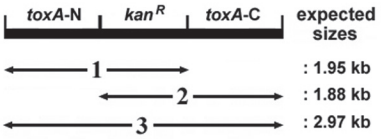

Figure 2. Diagram of PCR verification for genomic structure. The expected sizes and targets of three PCR approaches (1–3) were obtained.

P1 and P6 were used for PCR 1, P5 and P4 for PCR 2, and P1 and P4 for PCR 3.

In vitro expression of N-PMT

The expression of N-PMT in vitro was confirmed by a Western blot assay. Briefly, freshly prepared lysate of the PMT mutant was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). For immunodetection, the membrane was blocked in phosphate buffered saline (PBS) + 0.05% Tween-20 (pH 7.4) (PBST) containing 1% skim milk for 1 h. The membrane was washed 3 times with PBST before being incubated with the primary and secondary Abs for 1 h at 37°C. Anti-N-PMT mouse polyclonal Ab was produced by immunization of mice with recombinant N-PMT produced in *E. coli* (9), and used as a primary Ab. Goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated Ab (Serotec, Oxford, United Kingdom) was used as the secondary Ab. The membrane was developed in a diaminobenzidine (DAB) substrate buffer containing a DAB concentrate (Serotec) until brownish bands were observed. Color development was quenched by thorough washing in PBS.

Vaccination with PMT mutant

Pasteurella multocida toxin mutant bacterial cultures were prepared in BHI broth at a concentration of 1×10^8 cells/mL. Forty-eight 6-week-old ICR mice of both sexes (Charles River, Yokohama, Japan) were divided into 4 groups (Table II). Experimental groups were inoculated with 100 μL of freshly prepared culture (1×10^7 bacteria/mouse) and controls received 100 μL PBS. This was followed by 3 more booster injections with bacteria or PBS at 2-week intervals.

Ab induction in vivo

To check if partial truncated N-PMT expressed by PMT mutant in vivo could induce Abs, mouse blood samples were collected from the tail vein 7 d after the final booster injection and were used for an enzyme-linked immunosorbant (ELISA) assay. Briefly, 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with recombinant N-PMT (1 μg/well/90 μL) as previously described (9). The plates were blocked with PBS containing 1% skim milk and washed with PBST. The sera for IgG analysis were prepared at a 1:100 dilution (shown in preliminary experiments to be the optimal dilution). Ab in 90 μL PBST were incubated for 1 h at 37°C, emptied, and washed with PBST 3 times. The bound IgG was detected using goat anti-mouse HRP-conjugated IgG (Pierce, Rockford, Illinois, USA) diluted 1:500. The secondary Ab was incubated for 1 h at 37°C, emptied, and washed 3 times with PBST. A substrate solution consisting of 10 mL of 0.1 M citric acid buffer (pH 4.0), 250 μL of 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) stock solution (ABTS 100 mg ABTS in 4.5 mL distilled water), and 50 μL hydrogen peroxide (H_2O_2) was used to detect the presence of any bound secondary Ab. The plate was developed in the dark at room temperature for 15 min. The absorbance at 405 nm was read using a Multiskan EX ELISA reader (Thermo LabSystems, Beverly, Massachusetts, USA). The results were expressed as the average ± standard deviation *(s)* of the end-point titers. A *t*-test was used to examine the differences in the mean Ab values using GraphPad Instat Software 3.05 (GraphPad software, La Jolla, California, USA).

Bacterial and toxin challenges

For the preparation of bacterial toxin as previously described (8), 50 mL of wild type *P. multocida* overnight cultures (1×10^8 cells/mL) were washed twice with 10 mL PBS, sonicated, and the supernatants filtered through a 0.2-μm membrane filter. Fifteen days after vaccination, groups 1 and 3 were challenged with 500 μL of freshly prepared bacterial lysate (bacterial toxin, which corresponds to LD100), while groups 2 and 4 where challenged with 100 μL of freshly prepared wild type *P. multocida* culture (corresponds to LD100). The mortality rate was recorded for 10 d after the inoculation. A *t*-test was used to examine the differences in the protection rates.

Results

Disruption of *toxA* by homologous recombination

Figure 3 shows the results of the PCR verification. As expected, the 1.95 kb PCR1, 1.88 kb PCR2, and 2.97 kb PCR3 fragments were

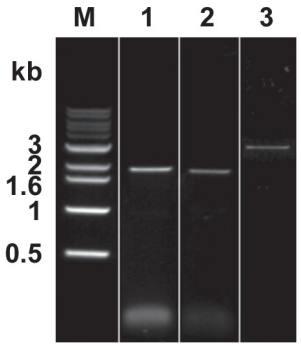

Figure 3. Results of PCR verification. PCR products with expected sizes (1.95 kb for PCR1, 1.88 kb for PCR2, and 2.97 kb for PCR3) were produced. Lane 1: PCR1, lane 2: PCR2, lane 3: PCR3.

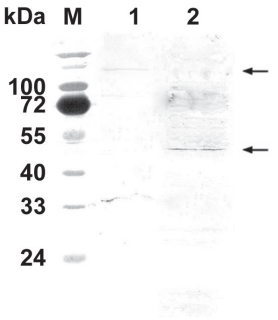

Figure 4. Antigenic recognition of N-PMT. Mouse polyclonal Ab raised against the recombinant N-PMT reacted with intact PMT (lane 1 upper arrow, approximately 146 kDa) and a bacterial protein of approximately 47 kDa (lane 2 lower arrow). M: molecular size marker.

Table III. Result of challenge studies using live bacterial culture or lysate

	Days after challenge					
Group	1	3	5	7	10	Total
1	—	—	—	—	2	2/12*
2	—	—	—	—	1	1/12*
3	2	10	—	—	—	12/12
4	—	6	—	—	6	12/12

* Two-sided *P*-value is lower than 0.0001, which is extremely significant on Fisher's exact test.

produced. The sequences of the PCR verification products were analyzed and confirmed by DNA sequencing (data not shown).

Expression of N-PMT in vitro

Anti-N-PMT mouse polyclonal Ab raised against recombinant N-PMT reacted with intact PMT from wild type *P. multocida* lysate and against a PMT mutant protein of approximately 47 kDa (Figure 4), confirming the in vitro production of a truncated N-PMT.

Ab induction in vivo

Mouse Abs against N-PMT expressed in vivo were successfully induced, as shown by an ELISA. Analysis of the mouse IgG level against N-PMT demonstrated that the mice, intraperitoneally immunized with the mutant *P. multocida*, successfully produced IgG (mean OD value = 1.472, $s = 0.195$, $P < 0.001$). However, no significant increase in IgG level was shown in the control group (mean OD value = 0.267, $s = 0.025$).

Bacterial and toxin challenge

Most of the mice immunized with mutant *P. multocida* were protected upon challenge (Table III). In total, 21 of the mice (87.5%) survived in the PMT mutant-immunized group, while none survived in the PBS control group ($P < 0.0001$).

Discussion

Recently, a genetically modified PMT toxin produced by replacing the serine at position 1164 with alanine, and the cysteine at position 1165 with serine led to a complete loss of the toxic effects of PMT without impairing the ability to induce protective immunity in pigs (13). Also, vaccination of sows with a mixture of 3 recombinant fragments of the PMT fragments (N-terminal, middle, and C-terminal) with/without intact *P. multocida* produced high levels of neutralizing Ab and protection of offspring against a PMT challenge (13). These suggest that the induction of IgG and/or IgA protective immunity against pasteurellosis can be achieved when the appropriate immunogens and administration routes are used.

Currently, clinical PAR is usually controlled by combined vaccination with *Bordetella bronchiseptica* and *P. multocida*, which consists mainly of toxoid and/or somatic antigens of both bacteria. Using these vaccines, pathogenic lesions, excretion of bacteria, and the time to market under experimental and field conditions are decreased. Some vaccines also have been shown to protect pigs against the development of lung lesions when administrated properly. Although vaccination has unquestionable beneficial effects, current vaccines are not able to eliminate the bacteria (14). Also, in our previous study (8), none of the mice vaccinated with a PMT-eliminated mutant (*toxA* knock-out) survived after wild type challenge, indicating that mouse Abs against outer structural and/or inner cytosolic proteins of *P. multocida* are not protective. So, the toxigenicity of this bacterium seemed to be derived mainly from PMT and the protective immunity against wild type *P. multocida* can be acquired only when protection against PMT has been established (13). Also, animals vaccinated with recombinant N-PMT are successfully protected after wild type challenge (9), which prompted us to develop a mutant expressing only N-PMT.

Expression of N-PMT in vitro and in vivo was confirmed by western blot and ELISA indicating that the elimination of the middle

and C-terminal fragments of PMT has no influence on protein expression. Mice immunized with mutant successfully produced anti-N-PMT Abs without any pathogenic effects of PMT, indicating this mutant has been well attenuated and N-terminal itself has little damage to animals. In the challenge experiments, only 3 out of 24 mice in groups 1 and 2 (12.5%) were killed, while all control mice succumbed. This indicates that protective Ab against PMT can be induced by purified (9) and/or bacterial (this study) recombinant N-PMT.

In this study, we constructed an attenuated *P. multocida* expressing only N-PMT to demonstrate the potential of this mutant to elicit a protective immune response in mice. Although further experiments with pigs will be performed, we conclude that this mutant represents a good candidate for the development of live bacterial vaccine.

Acknowledgment

The authors thank Ms. Myeonghwa Kim (College of Veterinary Medicine, Chonnam National University) for her excellent technical assistance.

References

1. de Jong MF. Progressive and nonprogressive atrophic rhinitis. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, eds. Diseases of Swine. Ames, Iowa: Iowa State Univer Pr, 1999:355–384.
2. Pijoan C. 1999. Pneumonic pasteurellosis. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, eds. Diseases of Swine. Ames, Iowa: Iowa State Univer Pr, 1999:511–520.
3. Hunt ML, Adler B, Townsend KM. The molecular biology of *Pasteurella multocida*. Vet Microbiol 2000;72:3–25.
4. Pullinger GD, Bevir T, Lax AJ. The *Pasteurella multocida* toxin is encoded within a lysogenic bacteriophage. Mol Microbiol 2004;51:255–269.
5. van Diemen PM, de Vries Reilingh G, Parmentier HK. Immune response of piglets to *Pasteurella multocidatoxin* and toxoid. Vet Immunol Immunopathol 1994;41:307–321.
6. Liao CM, Huang C, Hsuan SL, et al. Immunogenicity and efficacy of three recombinant subunit *Pasteurella multocida* toxin vaccines against progressive atrophic rhinitis in pigs. Vaccine 2006;24:27–35.
7. Hodgson JC, Finucane A, Dagleish MP, et al. Efficacy of vaccination of calves against hemorrhagic septicemia with a live *aroA* derivative of *Pasteurella multocida* B:2 by two different routes of administration. Infect Immun 2005;73:1475–1481.
8. Kim TJ, Lee JI, Lee BJ. Development of a *toxA* gene knock-out mutant of *Pasteurella multocida* and assessment of its protective effects. J Microbiol 2006;44:320–326.
9. Seo JY, Pyo HJ, Lee SM, et al. Expression of 4 truncated fragments of *Pasteurella multocida* toxin and their immunogenicity. Can J Vet Res 2009;73:184–189.
10. Ausubel FM, Brent R, Kingston RE, et al. Short Protocols in Molecular Biology. New York, New York: John Wiley & Sons, 2002.
11. Sambrook J, Fritsch EF, Maniatis T. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Lab Pr, 2001.
12. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 2000;97:6640–6645.
13. To H, Someno S, Nagai S. Development of a genetically modified nontoxigenic *Pasteurella multocida* toxin as a candidate for use in vaccines against progressive atrophic rhinitis in pigs. Am J Vet Res 2005;66:113–118.
14. Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A. Efficacy of vaccines against bacterial diseases in swine: What can we expect? Vet Microbiol 2004;100:255–268.